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(54) Title: FACTOR VIII DERIVATIVES

(57) Abstract

A new Factor VIII derivative comprising a functional A2 domain, in which one or more cysteine residues have been deleted or substituted by one or more other amino acid residues, said Factor VIII derivative shows a coagulant activity of Factor VIII which is stable for an extended period.

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TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
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VN	Viet Nam

:NSDOCID: <WO___9518827A1_I_>

TITLE

Factor VIII Derivatives.

FIELD OF THE INVENTION

The present invention relates Factor VIII derivatives comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues, said Factor VIII derivatives showing a coagulant activity of Factor VIII, a method for the preparation of Factor VIII derivatives comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues. Furthermore, the invention relates to pharmaceutical preparations comprising a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues substituted by a serine residue, and the use of a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues for the preparation of a pharmaceutical preparation for the treatment of diseases caused by an absence or deficiency of the Factor VIII of a subject.

BACKGROUND OF THE INVENTION

Haemophilia A is an X-chromosome-linked inherited disease which afflicts 1-2 males per 10,000. The disease is caused by an absence or deficiency of Factor VIII. Factor VIII is a large glycoprotein (native M_r 330000 - 360000), which is present in plasma at low concentrations (0.1 nM (Rapaport, West.J.Med. (1993) 158:153-161)). It is an essential element in the proteolytic cascade which converts soluble fibrinogen to insoluble fibrin, forming a clot to prevent blood loss

from traumatized tissue. In the bloodstream, it is found in noncovalent association with von Willebrand factor (vWF) which acts as a stabilizing carrier protein. Factor VIII is susceptible to cleavage by thrombin, activated protein C, plasmin, and other serine proteases. It is generally isolated from plasma or plasma products as a series of related polypeptides ranging from M_r 160000-40000 with predominant species of M_r 92000 and M_r 80000-77000. This complex pattern has made the analysis of the structure of active Factor VIII very difficult.

Factor VIII and the related polypeptides have been described by F. Rotblat et al, Biochemistry (1985) 24:4294-4300; G.A. Vehar et al, Nature (1984) 312:337-342; J.J. Toole et al, Nature (1984) 312:342-347; and M.A. Truett et al, DNA (1985) 4:333-349. The sequence has been reported by J.J. Toole et al, supra; W.I. Wood et al, Nature (1984) 312:330-336; and M.A. Truett et al, supra.

The full-length protein contains three repeats of the A-domain and two repeats of the C-domain together with a heavily glycosylated B-domain, ordered A1-A2-B-A3-C1-C2. The B-domain is not required for the function of Factor VIII (Burke et al. (1986) J.Biol.Chem. 261:12574-12578).

By thrombin activation, the heavy chain is cleaved between the A1 and the A2-domains C-terminal of Arg-372 and 41 amino acids are cleaved off from the N-terminus of the light chain C-terminal of Arg 1688.

Factor VIII has historically been isolated from blood in a concentrated form for therapeutic treatment of haemophilia. However, Factor VIII is only present in the blood in extremely small amounts and a vast number of donors have to be involved. Moreover, the purification process is laborious and expensive. Concerns regarding transmission of HIV and other blood-borne diseases as well as shortage of supplies have

especially stimulated activity to provide alternative supplies of Factor VIII, thus leading to the development of recombinant techniques.

The preparation of proteins having Factor VIII activity by recombinant techniques has inter alia been disclosed in a number of patent publications. Thus, European Patent Application No. 160 457 and International Patent Application No. WO 86/01961 disclose recombinant production of full length Factor VIII, European Patent Application No. EP 150 735 discloses a complex of subunits of Factor VIII having coagulant activity and recombinant production of subunits of Factor VIII, European Patent Application No. EP 232 112 and International Patent Application No. WO 91/07490 disclose co-expression of subunits for the production of complexes showing coagulant activity, and International Patent Application No. WO 86/06101, International Patent Application No. WO 87/04187, International Patent Application No. WO 87/07144, International Patent Application No. WO 88/00381, European Patent Application No. EP 251 843, European Patent Application No. EP 253 455, European Patent Application No. EP 254 076, U.S. Patent No. 4.980.456, European Patent Application No. EP 294 910, European Patent Application No. EP 265 778, European Patent Application No. EP 303 540, and International Patent Application No. WO 91/09122 disclose recombinant expression of Factor VIII having one or more deletions in the molecule, or binding to antibodies inhibiting Factor VIII.

The development of the recombinant techniques has provided a means for ensuring sufficient supplies of Factor VIII.

The lack of stability of Factor VIII still imposes difficulties on the storing and handling of Factor VIII giving rise to vast losses of Factor VIII proteins.

Fay Biochimica et Biophysica Acta(1987) 952:181-190 discloses a chemical crosslinking of Factor VIIIa giving a Factor VIIIa dimer composed of the 73000 and 51000 subunits comprising chemical "intra-chain crosslinks" and retaining more than 60% of its initial clotting activity. The resulting stabilized Factor VIIIa still had "a significant level of activity to allow the determination of the sedimentation coefficient" after 16 hours. However, there is no indication that the Factor VIIIa dimer prepared by Fay should retain coagulant activity for extended periods of time.

Thus, there is still a need for a method of stabilizing Factor VIII in order to ensure that as few units of Factor VIII as possible are lost during activation as well as storing and handling of Factor VIII and pharmaceutical preparations comprising the same.

In published International Patent application No. WO 88/00210 it is mentioned in a claim that one or more Cys-amino acids may be replaced by another amino acid, preferably serine, when combining an N-terminal fragment of Factor VIII:C with a molecular weight of 92 to 210 kD and a C-terminal fragment of Factor VIII:C with a molecular weight of 80 to 70 kD producing a coagulation active complex. In the specification there is no indication of the reason for carrying out such replacement nor the effect thereof.

It has now surprisingly been found that a Factor VIII derivatives comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by one or more other amino acid residues show improved stability and coagulant activity for extended periods of time both in vitro and in vivo.

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by one or more other amino acid residues, said Factor VIII derivative showing a coagulant activity of Factor VIII.

In another aspect, the invention relates to a method for the preparation of Factor VIII derivatives comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues.

In a further aspect, the invention relates to pharmaceutical preparations comprising a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues.

In yet another aspect, the invention relates to the use of a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues for the preparation of a pharmaceutical preparation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues, said Factor VIII derivative showing a coagulant activity of Factor VIII.

Preferably, a cysteine residue is substituted by an amino acid residue selected from the group consisting of alanine,

threonine, serine, glycine and asparagine, more preferred by a serine residue.

The coagulant activity is in the same order of magnitude as the coagulant activity of the corresponding "unsubstituted" Factor VIII and is maintained for an extended period of time as compared with the very rapid decline of the coagulant activity of Factor VIII after activation.

The amino acid sequence of the Factor VIII derivatives of the invention may correspond to the amino acid sequence of full length human Factor VIII as described above. The Factor VIII derivative may also have an amino acid sequence corresponding to a shortened form of Factor VIII having one or more deletions in the molecule, subunits of Factor VIII or complexes of subunits of Factor VIII provided that the Factor VIII derivative comprises the parts of the Factor VIII molecule being necessary for imparting the molecule coagulant activity per se or after activation using e.g. thrombin.

A complex may be held together by a ionic bridge or any other chemical binding imparting the complex coagulant activity. A ionic bridge may be a divalent metal bridge comprising e.g. calcium, cobalt or manganese ions.

In a preferred aspect of the invention, the Cys residue in position 692 of full length Factor VIII is substituted by a Ser residue.

A preferred Factor VIII derivative is a calcium-bound complex of the M_r 92000 and M_r 77/80000 doublet subunits of human Factor VIII wherein Cys 692 has been replaced with Ser.

In a further preferred aspect of the invention, the Factor VIII derivatives are derivatives in which also Glu 720 is deleted or substituted by another amino acid residue selected from the group consisting of Gln, Ser, Thr, Val and Ala.

In a further preferred aspect of the invention, the Factor VIII derivatives are derivatives in which also Tyr 729 is deleted or substituted by another amino acid residue selected from the group consisting of Val, Ala and Ile.

During recombinant production, the derivatives of Factor VIII wherein Glu 720 is deleted or substituted by another amino acid residue and/or wherein Tyr 729 is deleted or substituted by another amino acid residue show higher resistance against cleavage by enzymatic activity present in the medium which gives rise to an increased yield of the desired Factor VIII derivatives.

The invention also relates to a method for preparing a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues, comprising culturing a host cell transformed with a gene encoding the Factor VIII derivative under conditions wherein the gene is expressed and the expressed product secreted, and isolating the Factor VIII derivative.

In a preferred embodiment of the method of the invention, the Cys residue in position 692 of full length Factor VIII is substituted by a Ser residue.

In a further preferred embodiment of the invention, the Factor VIII derivative produced is in the form of a calcium-bound complex of the M_r 92000 and M_r 77/80000 doublet subunits of human Factor VIII wherein Cys 692 has been replaced with Ser.

In another embodiment of the invention, a Factor VIII derivative is produced wherein Glu 720 is deleted or substituted by another amino acid residue selected from the group consisting of Gln, Ser, Thr, Val and Ala.

In yet another embodiment of the invention, a Factor VIII derivative is produced, wherein Tyr 729 is deleted or substituted by another amino acid residue selected from the group consisting of Val, Ala and Ile.

The gene encoding the Factor VIII derivatives of the invention may be prepared from DNA encoding the corresponding human Factor VIII by conventional site specific mutagenesis. The DNA may be wholly or partly cDNA, chromosomal DNA and/or synthetic DNA.

The method of the invention may be carried out in a manner analogous to those described in the patent applications listed above. The resulting Factor VIII derivative may be purified by standard techniques.

In the alternative, the Factor VIII derivative may be produced from Factor VIII isolated from plasma by methods known per se, e.g. as described in EP patent No. 83483, EP patent No. 150735 or EP patent No. 197901 by deleting or substituting Cys 692 with Ser. Such substitution may be carried out by cleaving off a part of the A2 domain and coupling with a complementary fragment having the desired amino acid sequence. The complementary fragment may be produced by chemical synthesis or substituting the desired Cys residue in a fragment isolated from a plasma source in a manner known per se or produced by recombinant techniques.

The coupling may also be carried out by transesterification techniques introducing the desired complementary fragment into the remaining part of the A2 domain possessing a suitable leaving group by a manner known per se.

Furthermore, the invention relates to a gene encoding a Factor VIII derivative in which one or more cysteine residues has been deleted or substituted by one or more other amino acid residues. Such a gene comprises a DNA encoding said

human Factor VIII derivative and may comprise cDNA, chromosomal DNA and/or synthetic DNA.

In a further aspect, the invention relates to a pharmaceutical preparation comprising a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues in admixture with a parenterally acceptable vehicle or excipient.

A pharmaceutical preparation according to the invention may comprise further pharmaceutical excipients well known to those skilled in the art. These include, for example, various bulking agents, additional buffering agents, antioxidants, preservatives, stabilizers, and the like.

In a still further aspect, the invention relates to the use of a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues for the preparation of a pharmaceutical preparation for the treatment of diseases caused by an absence or deficiency of the Factor VIII of a subject.

In yet another aspect, the invention relates to a method for preventing or treating diseases caused by absence or deficiency of Factor VIII:C in a subject comprising administering to the subject a pharmaceutically active amount of a Factor VIII:C derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues in admixture with a pharmaceutically acceptable vehicle or excipient.

Such diseases may e.g. be haemophilia A, both patients suffering from lack of Factor VIII due to lack of production or induction of Factor VIII being inactive, and inhibitor patients developing antibodies to Factor VIII. The derivatization

itself may modify the epitope of Factor VIII recognized by the antibodies of an inhibitor patient and thus be used directly for treating the haemophilia and bypassing the inhibitor activity without having to take any special measures to neutralize or "by-pass" the antibodies. Furthermore, the Factor VIII derivatives of the invention may show prolonged in vivo activity.

The invention also relates to a method of preparing a pharmaceutical preparation comprising a Factor VIII:C derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues comprising mixing the Factor VIII derivative with pharmaceutically acceptable vehicle and/or excipient and forming a suitable dosis form of the pharmaceutical preparation.

A suitable dosis form may e.g. be a lyophilized powder to be reconstituted with water for injection. Such lyophilized powder may be presented in a vial or in a prefilled syringe or pen device, e.g. a dual chamber syringe. The Factor VIII derivatives of the invention may also be presented in the form of a solution to be used in e.g. a pen device.

As used herein the term "full length Factor VIII" designates the full molecule comprising the amino acid residues 1-2332 as disclosed in Nature (1984) 312:339.

As used herein the term "Factor VIII-HC", "heavy chain" or "HC" designates the A1-A2 repeats of Factor VIII comprising the amino acid residues 1-740 of full length Factor VIII as disclosed in Nature (1984) 312:341.

The term "Factor VIII-LC", "light chain" or "LC" as used herein designates the A3-C1-C2 repeats of Factor VIII comprising the amino acid residues 1649-2332 as disclosed in Nature (1984) 312:341.

The term "functional A2 domain" is used herein to designate a polypeptide having an amino acid sequence only deviating from the amino acid sequence of the A2 domain of human Factor VIII to an extent not having an adverse effect on the overall coagulant activity in terms of specific activity (International Units of Factor VIII activity per mg protein) and duration of the coagulant activity.

The term "host cell" is used to designate cells which may be employed when preparing the Factor VIII derivatives of the invention by recombinant methods. Such cells are preferably mammalian cells and include for example COS cells, Chinese hamster ovary (CHO) cells, mouse kidney cells, hamster kidney cells, HeLa cells HepG2 cells, or the like.

The term "other amino acid residue" is used in the present specification to designate a naturally occurring α-amino acid residue different from the amino acid residue present in the native polypeptide.

The invention is explained more in detail in the below Examples which illustrate the invention. They are not to be considered as limiting the scope of the invention being defined by the appended claims.

MATERIALS AND METHODS

Buffer A: 50 mM imidazole, 0.15 mM NaCl, 0.1% BSA, pH 7.4.

Chromogenic Assay

The activity of Factor VIII was measured in a chromogenic assay (Coatest, Chromogenix), as described by the manufacturers, except that all reactions were carried out at room temperature and that the incubation times were altered: Phospholipid, Factor IXa + Factor X, $CaCl_2$ and the diluted sample was incubated 15 min before adding the substrate + the

thrombin inhibitor, and the colour reaction was allowed to take place for 10 min.

EXPERIMENTAL PART

EXAMPLE 1

Expression of the M_r 92000 subunit of human Factor VIII wherein Cys 692 has been replaced with Ser and combination with the M_r 80000 subunit of human Factor VIII to form a complex showing coagulant activity.

In plasmid pSVF8-92 encoding the Factor VIII-HC as described in EP 232112 the codon encoding Cys in position 692 was replaced by a codon encoding Ser by site mutagenesis using the method described in Norris et al: Nucleic Acids Res. **11** p 5103-5112, 1983. The plasmid was transfected into COS 7 cells as described by Burke et al. J. Biol. Chem. **261** p 12574-12578, 1986. The transfected cells were grown as described by Burke et el, and culture medium was harvested after 24 hours and diluted 6 fold with buffer A, and 50µl was incubated with 50µl Factor VIII-LC prepared as disclosed in WO 88/00210 diluted to 17 U/ml in buffer A. 20 µl 1 mM mercaptoethanol and 14 µl 0.1 M $MnCl_2$ was added. After 18 hours incubation at 22°C, Factor VIII activity was confirmed by Coatest analysis.

EXAMPLE 2

Coexpression of the M_r 92000 subunit of human Factor VIII wherein Cys 692 has been replaced with Ser and the M_r 80000 subunit of human Factor VIII to form a complex showing coagulant activity.

Using the calcium phosphate technique (Graham and van der Eb, Virol (1973) 52:456-7) COS7 cells were cotransfected with a plasmid pSVF8-92 encoding the Factor VIII-HC as described in EP 232112 in which the codon encoding Cys in position 692 was

replaced by a codon encoding Ser by site mutagenesis as described in Norris et al (ibid) and a plasmid pSVF8-80. The resulting transformed cells were cultured in the same manner as described in WO 91/07490.

Media samples were collected after 24 hours expression at 37 °C and assayed for the contents of Factor VIII activity by chromogenic activity assay (Coatest). The results of double transfections are stated in the below Table also showing the results of a sample of a corresponding wild type complex not having the substitution.

Table

Sample	FVIII Activity mU/ml
HC - LC	58 50
HC (Cys_{692}→Ser) - LC	39 40

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): 2880
(G) TELEPHONE: 44448888
(H) TELEFAX: 44493256
(I) TELEX: 37304

(ii) TITLE OF INVENTION:
(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: DK

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 740 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: human Factor VIII

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal wherein Cys 692 is replaced with another amino acid residue

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1 5 10 15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20 25 30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65 70 75 80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
85 90 95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100 105 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115 120 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145 150 155 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
165 170 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
180 185 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
195 200 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
210 215 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225 230 235 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
245 250 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
260 265 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
275 280 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
290 295 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305 310 315 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
325 330 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
340 345 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
355 360 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
370 375 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385 390 395 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
405 410 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
420 425 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
435 440 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
450 455 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465 470 475 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
485 490 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
500 505 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
515 520 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
530 535 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545 550 555 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
565 570 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
580 585 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
595 600 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
610 615 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625 630 635 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
645 650 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
660 665 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
675 680 685

Ile Leu Gly Xaa His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
690 695 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705 710 715 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
725 730 735

Ile Glu Pro Arg
740

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 740 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: human Factor VIII

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal wherein Cys 692 is replaced with Ser

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1 5 10 15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20 25 30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65 70 75 80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
85 90 95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100 105 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115 120 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145 150 155 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
165 170 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
180 185 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
195 200 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
210 215 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225 230 235 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
245 250 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
260 265 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
275 280 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
290 295 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305 310 315 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
325 330 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
340 345 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
355 360 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
370 375 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385 390 395 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
405 410 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
420 425 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
435 440 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
450 455 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465 470 475 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
485 490 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
500 505 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
515 520 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
530 535 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545 550 555 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
565 570 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
580 585 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
595 600 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
610 615 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625 630 635 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
645 650 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
660 665 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
675 680 685

Ile Leu Gly Ser His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
690 695 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705 710 715 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
725 730 735

Ile Glu Pro Arg
740

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 684 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid residues 1649-2332 of human Factor VIII

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: C-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr
1 5 10 15

Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr
20 25 30

Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
35 40 45

His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser
50 55 60

Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro
65 70 75 80

Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr
85 90 95

Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu Leu Gly
100 105 110

Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg
115 120 125

Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr
130 135 140

Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys
145 150 155 160

Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln His His Met Ala
165 170 175

Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp
180 185 190

Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu
195 200 205

Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr
210 215 220

Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser
225 230 235 240

Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn
245 250 255

Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala
260 265 270

Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln
275 280 285

Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn
290 295 300

Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys
305 310 315 320

Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu
325 330 335

Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys
340 345 350

Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val
355 360 365

Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile
370 375 380

Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro
385 390 395 400

Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr
405 410 415

Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile
420 425 430

Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu
435 440 445

Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp
450 455 460

Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly
465 470 475 480

Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile
485 490 495

Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser
500 505 510

Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met
515 520 525

Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala
530 535 540

Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala
545 550 555 560

Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn
565 570 575

Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met Lys Val
580 585 590

Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr Ser Met Tyr
595 600 605

Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly His Gln Trp Thr
610 615 620

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
625 630 635 640

Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg
645 650 655

Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg
660 665 670

Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
675 680

CLAIMS

What is claimed is:

1. A Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by one or more other amino acid residues.

2. A Factor VIII derivative as claimed in claim 1, wherein the Cys residue in position 692 of full length Factor VIII is deleted or substituted by an amino acid residue selected from the group consisting of alanine, threonine, serine, glycine and asparagine.

3. A Factor VIII derivative as claimed in claim 2, wherein the Cys residue in position 692 of full length Factor VIII is substituted by a Ser residue.

4. A Factor VIII derivative as claimed in claim 3 in the form of a calcium-bound complex of the M_r 92000 and M_r 77/80000 doublet subunits of human Factor VIII wherein Cys 692 has been replaced with Ser.

5. A Factor VIII derivative as claimed in any of claims 1 - 4, wherein Glu 720 is deleted or substituted by another amino acid residue selected from the group consisting of Gln, Ser, Thr, Val and Ala.

6. A Factor VIII derivative as claimed in any of claims 1 - 5, wherein Tyr 729 is deleted or substituted by another amino acid residue selected from the group consisting of Val, Ala and Ile.

7. A method for preparing a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues

has been deleted or substituted by a one or more other amino acid residues, comprising culturing a host cell transformed with a gene encoding the Factor VIII derivative under conditions wherein the gene is expressed and the expressed product secreted, and isolating the Factor VIII derivative.

8. A pharmaceutical preparation comprising a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues in admixture with a parenterally acceptable vehicle or excipient.

9. Use of a Factor VIII derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues for the preparation of a pharmaceutical preparation for the treatment of diseases caused by an absence or deficiency of the Factor VIII of a subject.

10. A method for preventing or treating diseases caused by absence or deficiency of Factor VIII:C in a subject comprising administering to the subject a pharmaceutically active amount of a Factor VIII:C derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues in admixture with a pharmaceutically acceptable vehicle or excipient.

11. A method of preparing a pharmaceutical preparation comprising a Factor VIII:C derivative comprising a functional A2 domain in which one or more cysteine residues has been deleted or substituted by a one or more other amino acid residues comprising mixing the Factor VIII derivative with pharmaceutically acceptable vehicle and/or excipient and forming a suitable dosis form of the pharmaceutical preparation.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00008

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/755, C12N 15/12, C12N 15/57
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, BIOSIS, WPI, WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 9206999 (THE SCRIPPS RESEARCH INSTITUTE), 30 April 1992 (30.04.92), page 5, line 18 - line 29 --	1-9,11
X	WO, A1, 8800210 (NORDISK GENTOFTE A/S), 14 January 1988 (14.01.88), claim 4 --	1-9,11
A	EP, A1, 0294910 (GIST-BROCADES N.V.), 14 December 1988 (14.12.88), claim 7 -- --------	1-9,11

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 March 1995	27 -04- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

:NSDOCID: <WO___9518827A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00008

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 10
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

09/02/95

International application No.
PCT/DK 95/00008

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9206999	30/04/92	AU-A- 9069591	20/05/92
		JP-T- 6502541	24/03/94
		AU-A- 1757592	02/11/92
		CA-A- 2107100	28/09/92
		WO-A- 9217192	15/10/92
WO-A1- 8800210	14/01/88	AU-B- 607927	21/03/91
		AU-A- 7644887	29/01/88
		DE-D,T- 3788944	26/05/94
		EP-A,B- 0272304	29/06/88
		SE-T3- 0272304	
		JP-T- 1500514	23/02/89
		NO-B- 176244	21/11/94
EP-A1- 0294910	14/12/88	AU-A- 1809788	04/01/89
		IL-A- 86693	24/06/94
		US-A- 5171844	15/12/92
		WO-A- 8809813	15/12/88